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Morphohistopathological alteration in the gills and central nervous system in *Cyprinus carpio* exposed to lethal concentration of copper sulfate

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Abstract

Copper Sulfate ($CuSO_4$) is the most used in aquaculture as chemotherapeutic bath against bacterial, fungal and parasitic diseases but it is very toxic for fish so the goal of this study was to determine the lethal concentration of $CuSO_4$ and evaluate it is toxicity in the gill and central nervous system (brain and spinal cord) in *Cyprinus carpio*. Fish exposed to 0, 2.5, 5 and 10 mg/L for 24 hours, each concentration with three replication each have six fish. The mortality rate was 100% at concentration 10 mg/L, which represented lethal concentration, while medium lethal concentration (LC_{50}) was determined by Trevan method and it is 5mg/L. The fish with LC_{100} concentration exhibit abnormal respiration with gasping swimming, nervous sings with up down and stay at basin then die at 2-3 hours. The histopathological examination of the gills revealed circulatory disturbances, cellularity reaction, progressive and regressive alteration, this microscopic alteration was evaluated as semi-quantities analysis and there was variable significant ($P \leq 0.05$) in the pathological alteration and gill indexes between two treatments. In the brain and spinal cord, the lesions are represented by vasogenic edema, infiltration of inflammatory cells with atrophy in the neuronal body cells and hemorrhage. It is concluded from this study that the use of copper sulfate is within limited concentrations because increasing its concentration leads to fish toxicity, and it was observed that the gill tissue is more sensitive to toxicity than the central nervous system.

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Introduction

Copper is an essential trace element that has beneficial roles in organisms, it is required for connective tissue and hemoglobin synthesis, cellular metabolism, and respiration, as well as to it is roles in the action of enzymes such as tyrosinase, copper-zinc superoxide dismutase and cytochrome c oxidase, which are involved in critical of growth and maturation (1,2). Copper inter aquatic environment industrial activity, herbicide, supplemented fish diet as well as it considered an essential component in some chemotherapeutic agent as copper sulfate, it is widely used in fish culture as one of the broad chemotherapeutic agents against bacterial, fungal, parasitic diseases it will be used at concentration 0.3-2 mg/L (3,4), in addition it has bio-effects and ability to reduce the negative effects of free radical oxygen, stimulated antioxidant activity and reduce adverse effects of toxic agent as nano-zinc oxide in *C. carpio* (5,6). The permissible limited concentration of Cu ion in Iraqi water environment is 0.05 mg/l. As pollution concentration is 0.199 mg/l, a high concentration of Cu inters the body through the digestive tract, gills, and accumulation in the tissue lead to abnormal locomotor responses and adverse pathological effects in fish and other aquatic organisms (7-10). Delahaut *et al.* (11) reported the significant effects of Cu in gill electrolytes and cause reduction in sodium ions and

cause, an imbalance of osmoregulation in *C. carpio* and cause damage to gill, kidney, liver, and spleen architecture (12), blood lysis, and anemia in *Clarias lazera*, and enzyme activity disturbances (13,14). Although the toxic effects of copper sulfate are more clearly in the gill, liver, and kidney, it has mild effects on the brain and causes histopathological alteration in the telencephalon, mesencephalon layers, and cerebellum. Boareto *et al.* (15) revealed the neurotoxicity effects of copper in *Oreochromis niloticus*, which cause central nervous system injuries and reduces acetylcholine activity in the brain. Recently study demonstrated the toxic effects of copper on the lateral line, loss of sensation, and loss of olfactory function even at low concentrations ≥20 parts per million and for short periods of about 3 hours (16). As the result of acute toxic effects of Cu on freshwater fish even at low concentrations (17), that the toxicity of copper sulfate in the brain will be unknown, this study was performed to demonstrate the histopathological alterations of lethal concentration during short time exposure in the gill, brain and spinal cord in *C. carpio*.

Materials and methods

Ethical approve

Scientific Ethical Committee on Animal Experimentation at College of Veterinary of Medicine, University of Mosul, UM.VET.2021.049.

Experimental animals

Seventy-two *C. carpio* weight of 200±10 gm, were obtained from the hatchery in Erbil governorate, and transported to the fish laboratory in College of Agriculture at the University of Mosul. The fish were left in the tank with aerated, dechlorinated water during the acclimation period and fed with commercial feed. during the experiment period, the light cycle was regular at 12 hours light/ 12 hours dark, and dissolved oxygen 5 mg/l, the pH 7.5, and the temperature was continued at 20±1.5ºC (18,19).

Determine lethal concentration of $CuSO_4$

After one week, expanding acclimation to laboratory circumstances and determining the lethal and median lethal concentration of copper sulfate, fish were randomly divided into four groups. Each sex fish was placed at 70 L of aerated water and treated with stock variable concentration of $CuSO_4 \cdot 5H_2O$ 0, 2.5, 5, 10 mg/L for 24 hours according to the Trevan method (20). The behavior and clinical signs were reported, and dead, poisoned fish were removed immediately from the aquarium to collect the gills, brain, and spinal cord, sample. At the end of the experiment 24 hours, fish from the control group were treated with general anesthesia MS-222 at a concentration of 150 mg/l (21) to collect the samples from gills, brain, and spinal cord.

Microscopic examination

Gills and Brain were fixed with 10% formalin for 48 hours. For dehydration, the sample treated with series ethanol xylene was used for sample clearance then embedded in the paraffin and sectioning at 5µm then staining with routine stained Hematoxylin & Eosin.

Semi-quantities score scheme (SSS)

The semi-quantitative scheme was employed for determining the severity and degree of histopathological changes in the gills. This system involves (score value, importance factor, alteration index and reaction pattern indices). Bernet *et al.* (22) briefly explain the SSS. The histopathological alteration was an evaluation for each ten gills filament from blinded slide has score value (SV; 0, 2, 4, 6), the histopathological alteration ranging from circulatory disturbances, inflammatory reaction, regressive and progressive alteration all these alterations take importance factor (IF) ranging from 0-3, 0) mean reversible alteration and minimal importance) while, 3) mean irreversible alteration and minimal importance).The formula for calculating the Alteration Index (AI) was multiplying the score value by the importance factor, AI= SV×IF (22). Calculating the gills index (GI) is by summing all AI- related to each category.

Statistical analysis

The data of this study was statistical analysis by T test and Chi^2 square in the ($P \leq 0.05$). Both these analyses were completed using (23).

Results

Lethal concentration experiment: The result of this experiment was reported the lethal concentrations of $CuSO_4$ at 10 mg/l led to killing the fish, and mortality was 100% during 24 hours, while the concentration at five mg/l was the median lethal concentration which led to killing 50% of treated fish as in figure 1.

Clinical signs and fish behavior

The fish treated at a variable concentration of $CuSO_4$ exhibited variable nervous and respiratory signs according to the duration time of exposure. After half hour from fish treated with 10 mg/l, fish had abnormal respiration there was an increase and then a reducation in the opercula frequency, excessive mucus secretion appeared as thick thread (Figure 2) with abnormal and gasping swimming and fish were excited and exhibited nervous signs at the last time of exposure with up-down swimming, then they stay at the bottom and died.

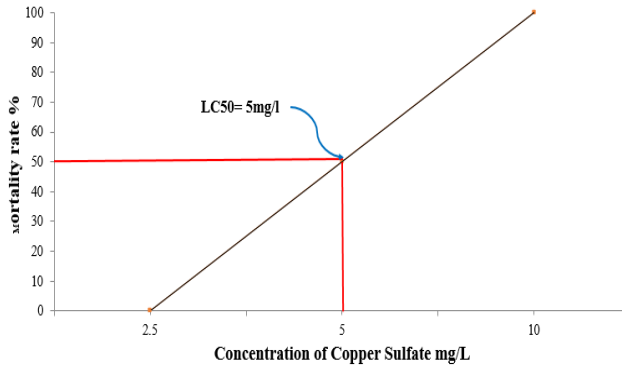


Figure 1: Standard curve for LC_{50} of copper sulfate (mg/L).

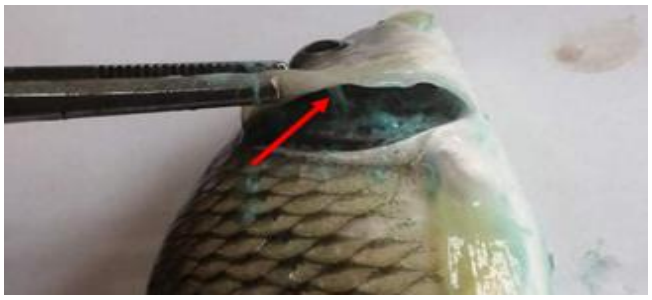


Figure 2: *C. carpio* exposed to lethal concentration of $CuSO_4$ 10 mg/l for three-hour show thick thread mucus secretion (red row**).**

Gross lesion

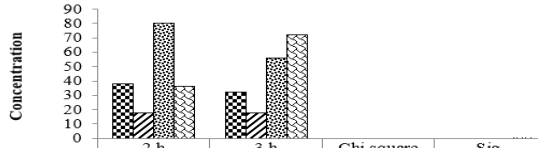
Excessive gill mucus secretion with $CuSO_4$ precipitated on the gill and body at a concentration 10 mg/L and less precipitation at 5mg/l with pale patches at the apex of secondary gill filaments with hemorrhage figure 3.

Figure 3: *C. carpio* exposed to lethal concentration of $CuSO_4$ 10 mg/l for three-hour show pale patches at the apex of secondary gill filaments (red row) and congestion (white row).

Semi-quantities score scheme

The histopathological alteration in the gill was estimated as semi- quantities exploration revealed significant alteration in both, treatments. The pathological lesion was classified into three categories. In the Circulatory alteration the statistical analysis revealed there was only a high significance ($P \leq 0.05$) in the mean of hyperemia in the gill of fish treated with $CuSO_4$ for three hours, this significance was detected in the progression alteration in the hypertrophy features in the epithelial, undifferentiated and chloride cells of gills in the fish treated for three hour and only hypertrophy of pillar cells was significant in the gills of fish treated for 2 hours in addition the third categories was a regressive alteration and the means of pathological lesions was significantly in both damage apex of primary gills filaments and lamellae necrosis in fish treated for three hours (Table 1).

The pathological alteration for each category was calculated as gills pathological index, which statistically revealed that fish treated for 3 hours is higher significant ($P \leq 0.05$) for both progressive and regressive indexes in contrast to circulatory and cellularity reaction figure 4.



	2 h	3 h	Chi square	Sig.
Gill Index Circulatory reaction	38	32	0.51	0.47
Gill Index Cellularity reaction	18	18	0.00	1
Gill Index Progressive reaction	80	56	4.24	0.04
Gill Index Regressive reaction	36	72	12.00	0.0005

Figure 4*:* Histogram for gills indexes.

Microscopic examination

The microscopic study of gills in the control fish shows the typical structure as in figure 5 in contrast to treated group, the primary lesions of fish gill which exposed to 10mg/l for 2 hour of $CuSO_4$ which represented by curling of the secondary gill filaments,, edema, lifting of the epithelial cells of secondary gill filaments figure 6, sever infiltration of mononuclear cell led to complete adhesion between secondary gill filaments with hypertrophy of mucus cells and vacuolar degeneration of undifferentiated cells figure 7, the lesion are more sever in the gills of fish exposed to lethal concentration for 3 hour represented by hyperemia in the arterial primary gill filaments figure 8 with abnormal shape of chloride cells with sever infiltration of inflammatory cells, hyperemic capillaries in the secondary gills filaments with vacuolar degeneration of pillar cells figure 9, edema and lifting epithelial cells of the secondary gills filaments and destruction the apex of primary gill gills filaments figure 10 and necrosis figure 11, microscopic examination of the gill arch revealed there was sever hyperemia with infiltration of inflammatory cells figure 12.

Table 1: Histopathological alteration in the gill of fish treated with lethal concentration of $CuSO_4$ 10 mg/l during 2 and 3 hours

Categories	Discerption	IF	Treatment	Mean	SD	T	sig
Circulatory	Edema	1	2	2.67	1.155	0.000	1.000
		3	3	2.67	1.155		
	Hyperemia	1	2	6.00	0.000	179.997	0.000
		1	3	0.00	0.000		
	Hemorrhage	1	2	4.00	2.000	1.732	0.158
		1	3	8.00	3.464		
Cellular reactive A- Progressive	Infiltration inflammatory cells	3	2	6.00	0.000	0.000	1.000
		3	3	6.00	0.000		
	Hydropic pillar cells	3	2	6.00	0.000	8.000	0.001
		1	3	0.67	1.155		
	Hydropic epithelial cells	3	2	2.00	0.000	599.010	0.000
		1	3	4.00	0.000		
	Hydropic undifferentiated cells	1	2	3.33	1.155	4.000	0.016
		1	3	6.00	0.000		
	Hypertrophy mucus cells	1	2	6.00	0.000	1.000	0.374
		3	3	6.00	0.000		
	Hypertrophy chloride cells	3	2	0.00	0.000	179.848	0.000
		3	3	6.00	0.000		
	Curling	1	2	10.00	6.928	1.808	0.145
		3	3	2.67	1.155		
	Epithelial lifting	1	2	18.00	0.000	1.000	0.374
		1	3	16.00	3.464		
Cellular reactive B-Regressive	Damage apex cells	1	2	0.00	0.000	537.988	0.000
		1	3	18.00	0.000		
	Adhesion	3	2	18.00	0.000	2.090	0.105
		3	3	18.00	0.000		
	Necrosis and dead lamellae	3	2	0.00	0.000	381.618	0.000
		1	3	18.00	0.000		

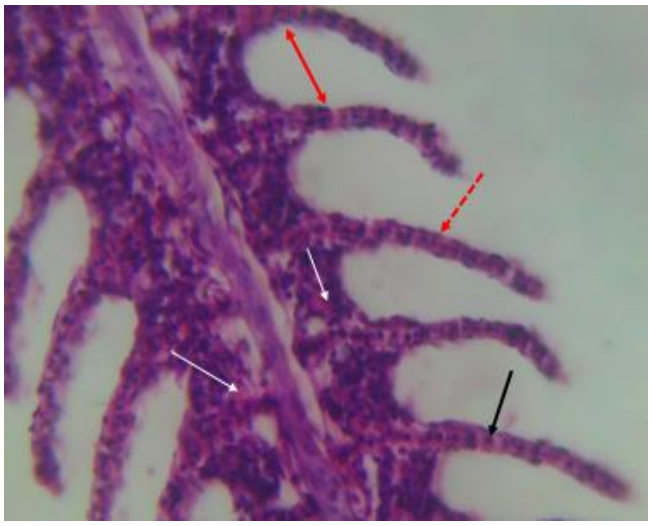


Figure 5: Microscopic examination of normal gill architecture in *C. carpio*, shows inter lamellar space (red-two head), secondary gill filament (red dot row), piller cells (black row), and mucus cells (white row). H&E. 270x.

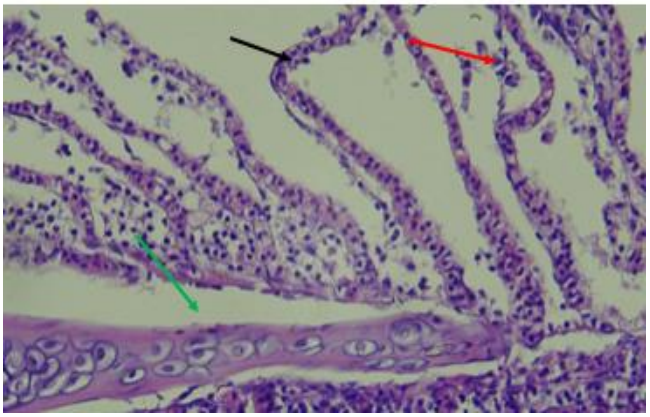


Figure 6: Microscopic examination of gill in fish exposed to lethal concentration 10 mg/l for 2 hour shows curling of the secondary gill filaments (black row), edema (green row), and lifting of the epithelial cells of secondary gill filaments (red row). H&E. 40x.

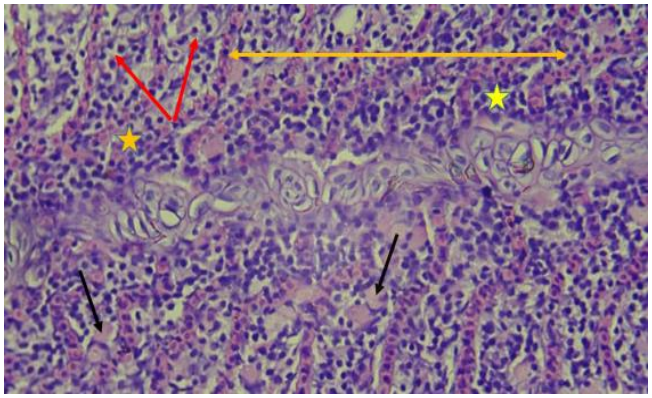


Figure 7: Microscopic examination of gill in fish exposed to lethal concentration 10 mg/l for 3 hour shows sever infiltration of mononuclear cell (yellow star) led to complete adhesion between secondary gill filaments (two head yellow row) with hypertrophy of mucus cells (black row), and vacuolar degeneration of undifferentiated cells (red row). H&E. 48x.

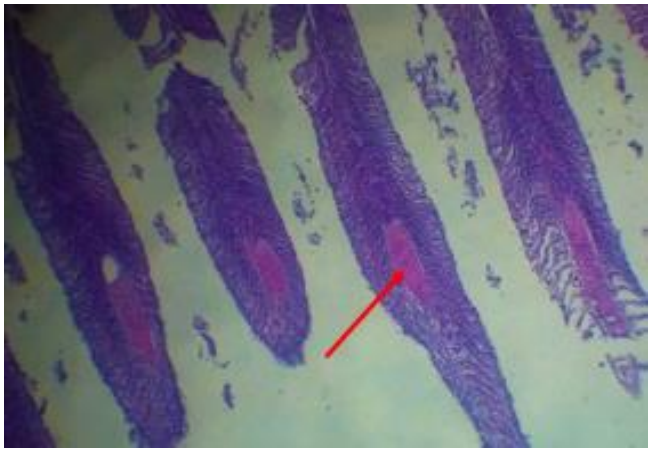


Figure 8: Microscopic examination of gill in fish exposed to lethal concentration 10 mg/l for 3 hour shows hyperemia in the arterial primary gill filaments (red row). H&E. 4x.

The effects of $CuSO_4$ in the central nervous system were clearly by microscopic examination in the brain and spinal cord. There was an infiltration of inflammatory cells in the brain with hemorrhage figure 13, hyperemia, vasogenic edema, with gliosis as in figure 14 and figure 15. In the spinal cord, the histopathological alteration distinguished as the infiltration of inflammatory cells with edema around neuronal bodies and tissue figure 16.

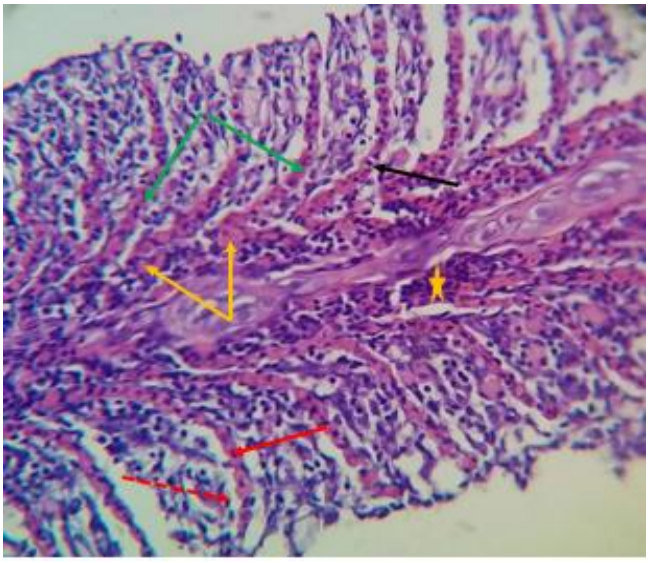


Figure 9: Microscopic examination of gill in fish exposed to lethal concentration 10 mg/l for 3 hour shows abnormal shape of chloride cells (yellow row) with sever infiltration of inflammatory cells (yellow star), hyperemic capillaries in the secondary gills' filaments (green row) with vacuolar degeneration of pillar cells (black row), edema (red row) with lifting epithelial cells (red dot row). H&E. 40x.

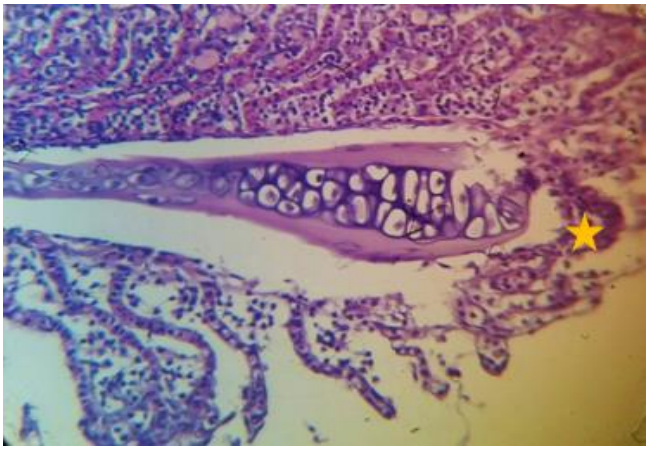


Figure 10: Microscopic examination of gill in fish exposed to lethal concentration 10 mg/l for 3 hour shows destruction the apex of primary gills filaments (yellow star). H&E. 40x.

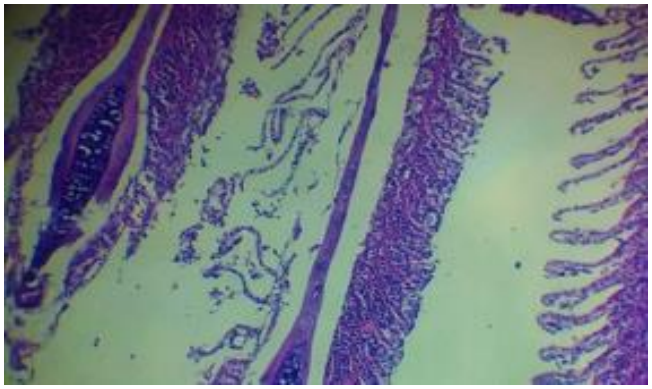


Figure 11: Microscopic examination of gill in fish exposed to lethal concentration 10 mg/l for 3 hour shows necrosis and dead gills. H&E. 13x.

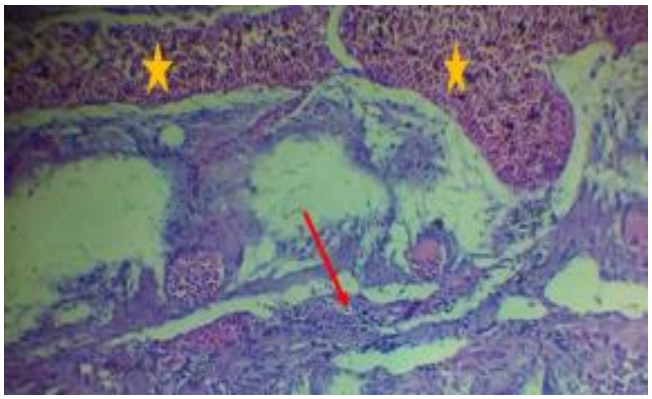


Figure 12: Microscopic examination of gill arch in fish exposed to lethal concentration 10 mg/l for 3 hour shows sever hyperemia (yellow star) with infiltration of inflammatory cells (red row). H&E. 16x.

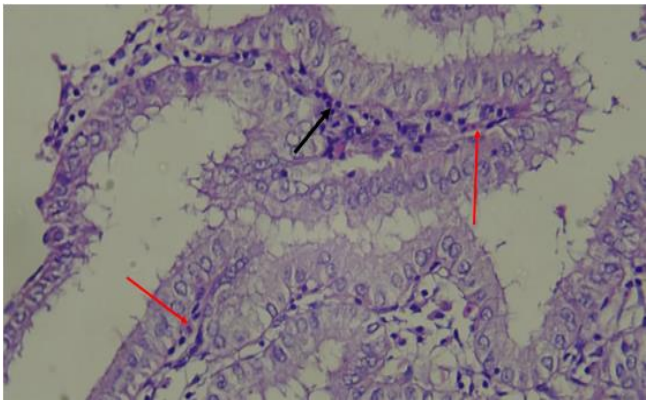


Figure 13: Microscopic examination of brain in fish exposed to lethal concentration 10 mg/l for 2 hour shows infiltration of inflammatory cells in the brain (black row) and hemorrhage (red row). H&E. 80x.

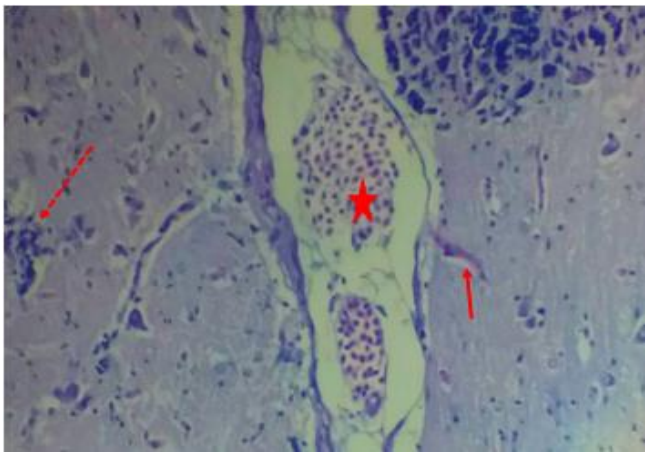


Figure 14: Microscopic examination of brain in fish exposed to lethal concentration 10 mg/l for 2 hour shows hyperemia (red star), vasogenic edema (red row) with gliosis (red dot row). H&E. 140x.

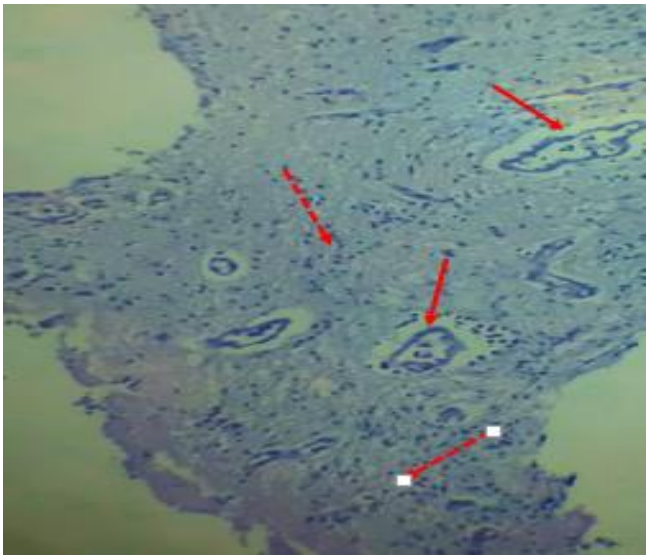


Figure 15: Microscopic examination of brain in fish exposed to lethal concentration 10 mg/l for 3 hour shows sever vasogenic edema (red row) with gliosis (red dot row). H&E. 240x.

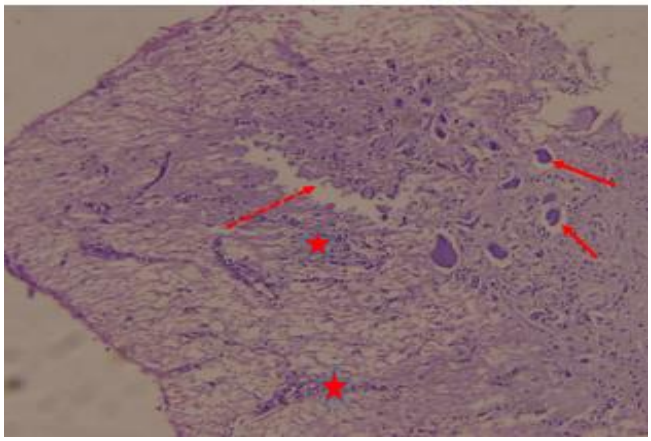


Figure 16: Microscopic examination of spinal cord in fish exposed to lethal concentration 10 mg/l for 3 hour shows infiltration of inflammatory cells (red star) edema around neuronal bodies (red row) and in the tissue (red dot row). H&E, 40X.

Discussion

Copper is essential elements for all organisms (24,25), copper sulfate is generally used in aquaculture as a chemotherapeutic agent. However, it is more toxic to *C. carpio* (26), so this study determined the LC_{100} of $CuSO_4$ in *C. carpio* is 10 mg/L, and LC_{50} is 5mg/L. This result does not agree with the results of (27), who reported the lethal concentration of $CuSO_4$ in *C. carpio* was 0.45 mg/L during 96 hours also, Nekoubin *et al.* (28) reported the LC_{50} is 2.422 mg/L in the grass carp *Ctenopharyngodon idella*, however the LC50 of $CuSO_4$ in freshwater fish variable from 0.30-7.57 ppm (29) These variable results may be related to fish species with different sensitivity to toxic material, age, size, physiological state, food habitat, water quality, and experimental conditions (30,31). The mortality occurred in the first hours of the experiments. This result comes with other results were got by Marinovic *et al.* (32), the death may result from direct effects of a toxic compound or indirect by forming an unconducive medium for the fish, the mucogenesis and increased mucus secretion from the gill may lead to decrease oxygen intake and cause respiratory disturbances under this condition fish may stress and exhibit abnormal behavior (32).

Histopathological alteration of gill architecture is considered a promising biomarker for lacking quality of fish environment (32-34) because it has direct contact with the aquaculture environment and has a specialized structure as thin epithelial cells with large particular sizable area. It is biological functions and ion balance. These alterations are common and represent general responses to variable stress such as season, the quantity of water, and infectious and non-infectious diseases. Thus, the semi-quantitive system score is a benefit for describing and analysis of the pathological alteration is considered a good indicator of the severity of lesions (11). Previous studies suggested the toxic, pathological effects of $CuSO_4$ in gills involve hypertrophy or hyperplasia in chloride cells, mucus and pillar cells, edema, hemorrhage, clubbing gill arch with thickening of primary and secondary lamellae led to fusion and dead tissue and necrosis. As a result, all of the lesions discovered in fish exposed to toxicants for a short period of 3 hours (35) and are likely to impair the respiratory, secretory, and excretory functioning of fish gills. Thirumavalavan (36) found that glucose and lactic acid levels in the blood of freshwater fish *Catla catla* exposed to copper sulfate were elevated. This elevation in glucose and lactic acid led to turnover cell respiration and caused an increase in anaerobic respiration and decreased in aerobic respiration. This is one of the causative agents for histopathological alteration in the gills and other organs. Also, a copper ion can share a sodium channel leading to competitive sodium ion uptake in the gill and affecting the Na^+-K^+/ATPase activity and disturbances the K and Ca ions balance. This is the key to cell injury. The alteration in the gill structure comes mainly from two hypotheses: firstly, it considers a defense mechanism (hypertrophy and hyperplasia) and increased respiratory surface with increased mucus secretion act as physical capture and reduce uptake xenobiotic, these lesions are reversible, while the second hypothesis considers the gill alteration as a pathological mechanism which are irreversible as disturbances in the blood circulation and necrosis (32). Boareto *et al.* (15), Al-Bairuty *et a*l. (37) and Sharma *et al.* (38) reported that copper sulfate is neurotoxic so this study suggested the toxicity of $CuSO_4$ in the brain and spinal cord which reported histopathological lesions represented by circulatory disturbances and infiltration of inflammatory cells. The neural tissue is vulnerable to oxidative damage which is the most well-known mechanism associated with copper ion toxicity, gliosis, and lacked Nissl substances with glycolysis leads to mitochondrial and microsomal dysfunctions (39-41).

Conclusion

This study suggested that copper sulfate is one of the chemotherapeutic agents and is commonly used in aquaculture. However, it should be used at a limited concentration with duration time because it is very toxic to fish, leading to respiratory disturbances, and abnormal fish behavior with histopathological alteration in both gill and CNS. This study suggested that gills are more susceptible to toxicity than CNS.

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Conflict of interest

No conflict of interest.

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التغييرات الشكلية والنسجية المرضية في الغلاصم والجهاز العصبي المركزي في اسماك الكارب الاعتيادي المعرضة للتركيز المميت من كبريتات النحاس

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الخلاصة

كبريتات النحاس هي الأكثر استخدامًا في تربية الأحياء المائية كحمام علاجي كيميائي ضد الأمراض الجرثومية والفطرية والطفيلية ولكنها شديدة السمية للأسماك لذا كان الهدف من هذه الدراسة هو تحديد التركيز المميت لكبريتات وتقييم سميته في الغلاصم والجهاز العصبي المركزي (الدماغ والحبل الشوكي) في اسماك الكارب الاعتيادي. تم تعريض الأسماك للتراكيز ٠ و ٢,٥ و ٥ و ١٠ ملغم/لتر ولمدة ٢٤ ساعة، ولكل تركيز ثلاث مكررات ولكل مكرر ست سمكات، كان معدل النفوق ١٠٠٪ عند التركيز ١٠ ملغم/لتر، والذي يمثل التركيز المميت، بينما تم تحديد التركيز المميت الوسطي بطريقة تريڤان وهو ٥ ملغم/لتر. أظهرت الأسماك المعرضة للتركيز المميت الكلي تنفس غير طبيعي وسباحة اصطياد الهواء مع علامات عصبية وصعود الى الأعلى ثم الاستقرار بقاع الحوض والنفوق خلال ٢-٣ ساعات. اظهر الفحص المرضي النسجي في الغلاصم االضطرابات الدورة الدموية، والتفاعل الخلوي والتغيير التراجعي والمتقدم، هذه التغيرات المجهرية تم تقييمها بالتحليل شبه الكمي وكان هنالك فرق معنوي (أ<٠,٠) في التغييرات المرضية ومؤشرات الغلاصم بين المعاملتين. تمثلت الآفات المرضية في الدماغ والحبل الشوكي بالوذمة الوعائية وارتشاح الخلايا الالتهابية مع ضمور جسم الخلايا العصبية والنزف. يستنتج من هذه الدراسة أن استخدام كبريتات النحاس يجب أن يكون بتراكيز محدودة لأن زيادة تركيزه يؤدي إلى تسمم الأسماك وقد لوحظ أن أنسجة الغلاصم أكثر حساسية للسمية من الجهاز العصبي المركزي.